*DEMONSTRATION OF THE UNIVERSALITY OF THE GENETIC CODE IN VIVO BY COMPARISON OF THE COAT PROTEINS SYNTHESIZED IN DIFFERENT PLANTS BY TOBACCO MOSAIC VIRUS RNA**

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Abstract.—The amino acid sequence of the coat protein of tobacco mosaic virus (TMV) synthesized in the garden zinnia (Compositae family) is compared with that synthesized in tobacco (Solanaceae family) by using the same preparation of TMV-RNA as messenger. The Compositae family is the most advanced group, and the Solanaceae family is a relatively advanced group in the plant kingdom. Both amino acid sequences are identical, including acetylserine as the N-terminal amino acid. This gives direct evidence that the RNA codon *in vivo* in the plant kingdom is universal between Compositae and Solanaceae families.

Although our knowledge of the genetic code is based largely on *in vitro* experiments with *Escherichia coli* systems,[1–8] evidence for its universality is strong. Many experiments with other bacteria,[9–11] plants,[12] and animals[13, 14] show that the same code is used by different organisms. However, the relative avidity of aminoacyl-transfer RNA to trinucleotides sometimes differs from species to species.[13] Von Ehrenstein *et al.*[15] have shown that radioactive amino acids are incorporated into rabbit hemoglobin molecules by rabbit reticulocyte polysomes even though they are presented as *E. coli* aminoacyl-transfer RNA conjugates.

On the other hand, protein synthesis in an *E. coli* cell-free system using natural messengers has been attempted, but unequivocal evidence for the synthesis of products characteristic of the messenger has been obtained only when bacteriophage RNA was used.[16, 17] Moreover, the fidelity of translation can be altered *in vivo* by extragenic suppressors[18, 19] and *in vitro* by altering conditions required for protein synthesis.[20–22] Thus, it is possible that different cells may differ in their RNA codon translation specificity.

The host range of some plant viruses is widely distributed in the plant kingdom. For example, tobacco (Solanaceae), larkspur (Ranunculaceae), spinach (Chenopodiaceae), French bean (Leguminosae), garden zinnia (Compositae), etc., are all susceptible to tobacco mosaic virus (TMV). If certain codons *in vivo* are not universal, the amino acid sequence of coat protein of TMV synthesized in one species might be different from that synthesized in another when the same virus RNA acts as the messenger.

In the present report, the amino acid sequence of the coat protein of TMV synthesized in garden zinnia is compared with that synthesized in tobacco using the same preparation of TMV-RNA as messenger. Garden zinnia belongs to the Compositae family, which is the most advanced phylogenic and probably evolutionary group. Tobacco is the most common host for TMV and belongs to the Solanaceae family, also a relatively advanced group in the plant kingdom.

Therefore, these two plants are good hosts to consider in an investigation of the universality of the genetic code using a plant-TMV system. Our results showed that amino acid sequences were identical, including acetylserine as the N-terminal amino acid. We submit this as direct evidence that the RNA codon *in vivo* is universal between tobacco and garden zinnia.

Materials and Methods.—A common Japanese strain of tobacco mosaic virus, TMV-OM, was used. The amino acid sequence had already been determined by Nozu and Okada.[23] Two amino acid exchanges were observed between OM and U1 (American strain) or Vulgare (German strain).

The virus was prepared from systemically infected leaves of *Nicotiana tabacum* var. Bright Yellow by the differential centrifugation method.[24] TMV-RNA was prepared by the bentonite-phenol method,[25] and the coat protein was isolated by the acetic acid method.[26]

Isolation of TMV from garden zinnia: Garden zinnia (*Zinnia elegans*) grown in the greenhouse was inoculated with TMV-RNA (100 μg/ml) by a glass spatula on carborundum-dusted leaves. About 3 weeks after inoculation, symptoms appeared and the leaves were harvested. Acetone-dried powders were prepared by placing leaves of garden zinnia in a Waring Blendor, covering them with at least 5 vol of chilled acetone, and blending vigorously for 2 min. The resulting slurry was filtered through a Büchner funnel and washed with an excess of chilled acetone; the residue was spread out on a filter paper and allowed to dry in a cold room. The acetone powder was extracted with approximately 5 to 10 vol of solvent, pH 7.1 (0.1 *M* sodium phosphate buffer, 0.01 *M* Versene, 0.5% ascorbic acid (w/v)). The crude extract was filtered through four layers of cheesecloth, and the filtrate was centrifuged at low speed (10,000 $\times$ *g*, 15 min), followed by high-speed centrifugation (100,000 $\times$ *g*, 2 hr) to collect the virus. In order to remove chlorophyll from the preparation, the pellet was suspended in the same solvent containing nonionic detergent, Triton X-100 (final concentration, 5%), followed by the low-speed centrifugation. The virus solution was purified by the differential centrifugation.

Separation of tryptic digest of coat protein: Coat protein was digested with TPCK-treated trypsin at 37°C for 3 hr at pH 8 with an autotitrator. The reaction mixture was adjusted to pH 4.5 with acetic acid and centrifuged to remove the insoluble core, which was purified by salting-out with a one-third saturation of ammonium sulfate and dialyzed against the distilled water overnight. The pH 4.5 soluble fraction was applied to a 0.9 $\times$ 150-cm column of Dowex 1 $\times$ 2 and eluted at 35°C with a gradient of pyridine-collidine-acetic acid buffer as described by Funatsu.[27] The flow rate was 40 ml/hr and 4 ml of eluate was collected per tube. Aliquots (0.5-ml) of each tube were assayed by use of the Folin-Lowry method.[28] Samples of each peak were hydrolyzed with constant-boiling HCl at 108°C for 24 hr and analyzed in a Yanagimoto automatic amino acid analyzer LC-5.

Isolation of N-terminal amino acid of protein: The protein (50 mg) was digested with 0.5 mg chymotrypsin at 37°C for 2 hr at pH 7.8 with an autotitrator. The reaction mixture was finally adjusted to pH 3.8, and the resulting precipitates were centrifuged off. The supernatant was applied to a 0.9 $\times$ 10-cm column of Dowex 50 (H^+ form) and eluted at room temperature with distilled water at a flow rate of 40 ml/hr. Each fraction (4 ml) was analyzed spectrophotometrically between 220 mμ and 300 mμ, using the Hitachi spectrophotometer EPS-3. N-terminal peptide, which showed marked ultraviolet absorption between 260 and 280 mμ, was eluted immediately after the void volume.

^{14}C-acetylserine: ^{14}C-acetylserine was synthesized from ^{14}C-acetic anhydride and serine by the method of Narita.[29] This gave only one spot on a paper chromatogram (*n*-butanol:acetic acid:water = 4:1:1), and it is negative in the ninhydrin reaction.

Results.—In garden zinnia, TMV multiplied systemically and often produced symptomatologically chlorotic spots on the leaves.

TMV isolated from garden zinnia shows the same characteristic ultraviolet

absorption curve and infectious properties as that isolated from tobacco. TMV protein isolated from garden zinnia had the same amino acid composition as that isolated from tobacco.

Separation and amino acid analysis of the tryptic peptides: In order to compare the primary structure of TMV protein isolated from garden zinnia with that isolated from tobacco, the soluble fraction of the tryptic digest was chromatographed on a Dowex 1 × 2 as shown in Figure 1. The resulting elution pattern was identical with that of tryptic peptides of TMV isolated from tobacco previously reported by Nozu and Okada.[23] The amino acid compositions of the tryptic peptides are listed in Table 1. The composition of each was found to be the same as that of OM isolated from tobacco. In a comparison of the compositions in T10 and T12 with that of U1, exchanges were observed of isoleucine to valine and threonine to asparagine, respectively. These are the characteristic exchanges of the OM strain.[23]

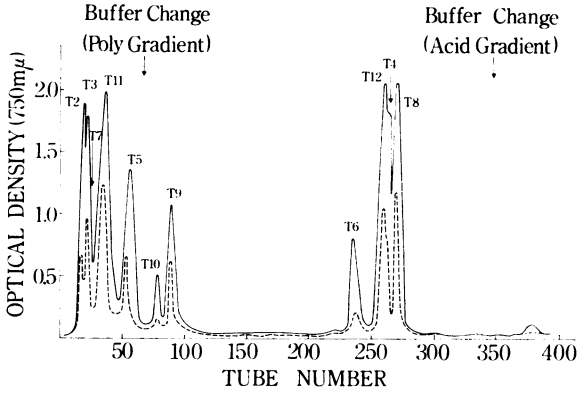

FIG. 1.—Chromatography of the pH 4.5-soluble peptides of TMV protein isolated from garden zinnia. The tryptic peptide mixture was applied on the Dowex 1 × 2 column (0.9 × 150 cm) equilibrated with pyridine-collidine-AcOH buffer (pH 8.2) and eluted at 35°C with the polygradient system described by Funatsu.[27] The flow rate was 40 ml/hr and 4 ml of eluate was collected. Aliquots (0.5 ml) of each tube were assayed by use of the Folin-Lowry method, the absorbance at 750 mμ being measured.

Elution pattern (*dotted line*) of the tryptic peptides of TMV protein isolated from tobacco[23] under the same conditions is superimposed for comparison.

The amino acid composition of T1 (core) was also identical to that isolated from tobacco. Moreover, the T1 was digested with chymotrypsin and followed by the chromatography on a Dowex 1 × 2. The elution pattern and amino acid composition of all peptides were the same as of that isolated from tobacco.

Identification of acetylserine at the N-terminus: N-terminal peptide was ninhydrin-negative and found to be composed of Ser_1, Tyr_1. By the carboxypeptidase A digestion, only tyrosine was liberated but free serine was not detected. In order to confirm that N-terminal serine residue is acetylated, the digests were cochromatographed with ^{14}C-acetylserine on a Dowex 1 × 2 column as shown in Figure 2. Peak *b* overlapped with ^{14}C-acetylserine, and the acid hydrolysate

TABLE 1. *Amino acid composition of tryptic peptides of TMV protein isolated from garden zinnia.*

Peptide	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	Total‡
Asp	4.0(4)	...	...	2.0(2)	...	3.0(3)	1.0(1)	3.0(3)	2.0(2)	2.1(2)	1.0(1)	1.2(1)	19(19)
Thr	3.6(4)	0.8(1)	1.0(1)	...	...	1.9(2)	...	4.0(4)	0.9(1)	...	1.1(1)	1.0(1)	15(15)
Ser	4.2(5)	...	2.0(2)	1.1(1)	...	...	...	...	...	1.1(1)	1.2(1)	5.1(6)	16(16)
Glu	5.6(6)	1.0(1)	2.7(3)	...	...	...	...	4.4(4)	...	1.2(1)	...	1.0(1)	16(16)
Pro	2.2(2)	...	1.9(2)	1.1(1)	...	1.1(1)	...	1.2(1)	...	...	...	1.0(1)	8(8)
Gly	1.3(1)	...	...	...	...	1.2(1)	...	...	...	...	2.0(2)	1.9(2)	6(6)
Ala	3.5(4)	...	...	...	...	3.2(3)	...	2.9(3)	1.9(2)	1.1(1)	...	1.0(1)	14(14)
Val	1.4(1)	1.2*(2)	2.4*(3)	...	1.0(1)	1.9(2)	...	0.9(1)	1.9(2)	2.2(2)	...	0.8(1)	15(15)
Ile	2.5(3)	...	...	...	...	...	...	1.3*(2)	1.0(1)	2.1(2)	...	...	8(8)
Leu	3.8(4)	...	...	...	...	4.1(4)	...	1.0(1)	...	2.4(2)	...	1.0(1)	12(12)
Tyr	1.0(1)	...	...	...	0.7(1)	0.7(1)	...	...	...	...	0.8(1)	...	4(4)
Phe	2.7(3)	...	1.1(1)	2.2(2)	...	1.2(1)	...	...	...	...	...	1.1(1)	8(8)
Trp	†(1)	...	†(1)	...	...	...	...	...	...	...	...	†(1)	3(3)
Lys	...	...	1.0(1)	1.0(1)	...	...	...	...	...	...	...	...	2(2)
Arg	1.0(1)	1.0(1)	1.0(1)	...	1.0(1)	0.6(1)	1.0(1)	1.2(1)	2.0(2)	1.0(1)	1.0(1)	...	11(11)
$CysO_3$	1.0(1)	...	...	...	...	...	...	...	...	...	...	...	1(1)

Each peptide was hydrolyzed for 24 hr and analyzed for amino acid composition. The values in parentheses indicate the amino acid compositions of TMV protein isolated from tobacco.

* The low recoveries of Ile and Val are attributed to their resistance to hydrolysis.

† Tryptophan was detected qualitatively by the reaction of these peptides with Ehrlich's reagent.

‡ The total amino acid composition is shown as the sum total of the nearest number of residues per mole of each peptide.

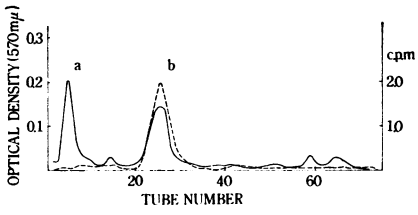

FIG. 2.—Cochromatography of N-terminal amino acid with ^{14}C-acetylserine. Carboxypeptidase A digests of N-terminal peptide were mixed with ^{14}C-labeled N-acetylserine and applied to 0.9 × 30-cm column of Dowex 1 × 2 equilibrated with 1 *M* acetic acid.

Elution was carried out at room temperature with acid gradient (10, 20, and 30% acetic acid, 200 ml each). The flow rate was 40 ml/hr and 4 ml of eluate was collected. Aliquots of each tube were assayed by ninhydrin reaction after alkaline hydrolysis.

Radioactivity of each fraction was measured by a gas flow counter (*dotted line*).

was identified as serine. Then peak *b* was found to be acetylserine and peak *a*, tyrosine. Consequently, the sequence of the N-terminal peptide of TMV protein isolated from garden zinnia was found to be acetyl-Ser-Tyr, identical to that isolated from TMV.

These results establish that the primary structures of the TMV protein synthesized in tobacco and garden zinnia are identical.

Discussion.—The biosynthesis of proteins involves the formation and stepwise elongation of peptide chains commencing from the N-terminal amino acid and under the genetic control of the base sequence in messenger RNA. The genetic code elucidated *in vitro* with synthetic nucleotides appears to be common among different organisms.[1–14] Little is known about *in vivo* codons, however, except those of *E. coli*[30, 31] and T4 phage-infected *E. coli*.[32, 33]

To confirm whether different cells translate the same viral RNA genetic message identically, we compared the amino acid sequence of TMV protein synthesized in tobacco (Solanaceae) and garden zinnia (Compositae) cells under the direction of the same TMV-RNA. The results showed that the two amino acid sequences were identical, indicating that the same *in vivo* codon is utilized by both tobacco and garden zinnia. These results provide direct support for the theory of *in vivo* universality of the genetic code among different families of plants.

The Compositae family, to which the garden zinnia belongs, is the most advanced group, and the Solanaceae family, which is a common host of TMV, is a relatively advanced group, in the phylogenic sense, among the families of plants. The Chenopodiaceae family, to which spinach belongs, is the least advanced group among the plants susceptible to systemic infection with TMV. When the amino acid sequence of TMV protein synthesized in spinach has been determined, we shall be able to say more concerning the universality of the *in vivo* codon in the plant kingdom. This work is now in progress in our laboratory.

Atherton recently reported[34] the formation of a particle similar to TMV in infectivity, morphology, and serology when an animal cell in culture is supplied with TMV-RNA, although the amino acid sequence is not determined. This system will also be very interesting to demonstrate the universality of the genetic code *in vivo*.

A mechanism of initiation of protein biosynthesis involving N-formylmethionyl-transfer RNA appears to be common in bacteria.[35–37] However, little is known so far concerning the corresponding process in other organisms. There is some

evidence to suggest that initiation of protein synthesis in animal cells[38, 39] differs from that in *E. coli*. According to our findings, the N-terminal group of both TMV proteins (tobacco and garden zinnia) is acetylated. These results may indicate that the initiation mechanisms of protein synthesis in tobacco and garden zinnia are identical. It remains unknown whether protein synthesis in plants is initiated by acetylated amino acid or whether the acetylation of N-terminal amino acid occurs after protein synthesis.

Summary.—The amino acid sequence of the coat protein of TMV synthesized in garden zinnia is compared with that synthesized in tobacco when the same preparation of TMV-RNA is used as messenger. The two amino acid sequences are identical, including acetylserine as the N-terminal amino acid. It appears that the *in vivo* codons in tobacco and garden zinnia are the same.

Abbreviations: TPCK, 1-(1-tosylamino-2-phenyl) ethyl chloromethyl ketone; TMV, tobacco mosaic virus.

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[1] Nirenberg, M., and P. Leder, *Science*, **145**, 1399 (1964).
[2] Leder, P., and M. Nirenberg, these PROCEEDINGS, **52**, 420 (1964).
[3] Trupin, J., F. Rottman, R. Brimacombe, P. Leder, M. Bernfield, and M. Nirenberg, these PROCEEDINGS, **53**, 807 (1965).
[4] Nirenberg, M., P. Leder, M. Bernfield, R. Brimacombe, J. Trupin, F. Rottman, and C. O'Neal, these PROCEEDINGS, **53**, 1161 (1965).
[5] Soll, D., E. Otsuka, D. Jones, R. Lohrmann, H. Hayatsu, S. Nishimura, and H. G. Khorana, these PROCEEDINGS, **54**, 1378 (1965).
[6] Nishimura, S., D. S. Jones, and H. G. Khorana, *J. Mol. Biol.*, **13**, 302 (1965).
[7] Nishimura, S., D. S. Jones, E. Ohtsuka, H. Hayatsu, T. M. Jacob, and H. G. Khorana, *J. Mol. Biol.*, **13**, 283 (1965).
[8] Kossel, H., A. R. Morgan, and H. G. Khorana, *J. Mol. Biol.*, **26**, 449 (1967).
[9] Groves, W. E., and E. S. Kempner, *Science*, **156**, 387 (1967).
[10] Protass, J. J., J. F. Speyer, and P. Lengyel, *Science*, **143**, 1174 (1964).
[11] Sager, R., I. B. Weomstein, and Y. Ashkenazi, *Science*, **140**, 304 (1963).
[12] Basilio, C., M. Bravo, and J. E. Allende, *J. Biol. Chem.*, **241**, 1917 (1966).
[13] Marshall, R. E., C. T. Caskey, and M. Nirenberg, *Science*, **155**, 820 (1967).
[14] Gupta, N. K., *J. Biol. Chem.*, **243**, 4959 (1968).
[15] Von Ehrenstein, G., and F. Lipmann, these PROCEEDINGS, **48**, 1424 (1962).
[16] Nathans, D., G. Notani, J. H. Schwarz, and N. D. Zinder, these PROCEEDINGS, **48**, 1424 (1962).
[17] Nathans, D., *J. Mol. Biol.*, **13**, 521 (1965).
[18] Weigert, M. G., and A. Garen, *J. Mol. Biol.*, **12**, 448 (1965).
[19] Brenner, S., A. O. W. Stretton, and S. Kaplan, *Nature*, **206**, 994 (1965).
[20] Ochoa, M., Jr., and I. B. Weinstein, these PROCEEDINGS, **52**, 470 (1964).
[21] Szer, W., and S. Ochoa, *J. Mol. Biol.*, **8**, 823 (1964).
[22] Davies, J., W. Gilbert, and L. Gorini, these PROCEEDINGS, **51**, 883 (1964).
[23] Nozu, Y., and Y. Okada, *J. Mol. Biol.*, **35**, 643 (1968).
[24] Boedker, H., and N. S. Simmons, *J. Am. Chem. Soc.*, **80**, 2550 (1958).
[25] Fraenkel-Conrat, H., B. Singer, and A. Tsugita, *Virology*, **14**, 54 (1961).
[26] Fraenkel-Conrat, H., *Virology*, **4**, 1 (1957).
[27] Funatsu, G., *Biochemistry*, **3**, 1351 (1964).
[28] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[29] Narita, K., *Biochim. Biophys. Acta*, **30**, 352 (1958).
[30] Berger, H., W. J. Brammar, and C. Yanofsky, *J. Mol. Biol.*, **34**, 219 (1968).
[31] Weigert, M. G., E. Gallucci, E. Lanka, and E. Garen, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), p. 145.

[32] Streisinger, G., Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, and M. Inouye, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), p. 77.
[33] Terzaghi, E., Y. Okada, G. Streisinger, J. Emrich, M. Inouye, and A. Tsugita, these PROCEEDINGS, **56**, 500 (1966).
[34] Atherton, J. G., *Arch. Ges. Virusforsch.*, **24**, 406 (1968).
[35] Capecchi, M. R., these PROCEEDINGS, **55**, 1517 (1966).
[36] Webster, R. E., D. L. Engelhardt, and N. D. Zinder, these PROCEEDINGS, **55**, 155 (1966).
[37] Marker, K., and F. Sanger, *J. Mol. Biol.*, **8**, 835 (1964).
[38] Narita, K., I. Tsuchida, and K. Ogata, *Biochem. Biophys. Res. Commun.*, **33**, 430 (1968).
[39] Arnstein, H. R. V., and H. Rahamimoff, *Nature*, **219**, 942 (1968).